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Award Number: DAMD17-01-1-0246

TITLE: Fas/FasL System in c-Myc Expressing Mammary Carcinoma
Cells

PRINCIPAL INVESTIGATOR: Christine M. Coticchia

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20007

REPORT DATE: June 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20050415 109

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE June 2004	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Jun 2003 - 31 May 2004)	
4. TITLE AND SUBTITLE Fas/FasL System in c-Myc Expressing Mammary Carcinoma Cells			5. FUNDING NUMBERS DAMD17-01-1-0246	
6. AUTHOR(S) Christine M. Coticchia				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Georgetown University Washington, DC 20007 E-Mail: coticchc@georgetown.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) The c-Myc oncogene is amplified or overexpressed in the majority of human breast cancers where it contributes to genomic instability, abrogation of cell cycle check points, cell proliferation, and apoptosis. In search of survival pathways that abrogate c-Myc's apoptotic function, our lab has uncovered a novel survival-signaling pathway that regulates Akt activation. In mammary carcinoma cells that overexpress c-Myc, calcium and calmodulin mediate EGF-induced activation of Akt. Inhibition of calmodulin with W7 leads to a rapid abrogation of ligand initiated activation of Akt. This phenomenon was observed in a number of mouse mammary carcinoma cell lines and human mammary epithelial cells regardless of c-Myc expression, however only c-Myc overexpressing cells underwent apoptosis when calmodulin was inhibited by W7, suggesting that calmodulin was providing a fundamental survival signal in those cells. Calmodulin and calmodulin-dependent kinase's (CaM kinases) role in the mammary epithelium is virtually unknown. A novel CaM kinase, Pnck (pregnancy upregulated nonubiquitous CaM kinase) was recently discovered and its expression profile has been described. Pnck is upregulated in the mouse mammary gland during development and in late stage pregnancy in a subset of epithelial cells that appear to be undergoing differentiation. Pnck is again upregulated in the mouse mammary glands undergoing postlactational involution, suggesting that Pnck expression is correlated with differentiation and apoptosis. In human breast tumors, Pnck expression was upregulated, but not in adjacent benign tissue, as well in a subset of human breast cancer cell lines. Interestingly, in mouse mammary tumors, Pnck was upregulated in MMTV-c-Myc derived tumors, but not other MMTV-oncogene derived tumors, suggesting that Pnck may be expressed in a c-Myc associated manner. Since our recent findings suggest that calcium and calmodulin play a fundamental role in survival signaling and may protect c-Myc overexpressing cells from apoptosis and Pnck is a CaM kinase upregulated in c-Myc-derived tumors, we have cloned the human Pnck gene and are characterizing Pnck's role in breast cancer cells. We propose that Pnck may influence the survival pathway in breast cancer. We aim to uncover Pnck's biological effect in breast cancer and to investigate its regulation.				
14. SUBJECT TERMS No subject terms provided.			15. NUMBER OF PAGES 22	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

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INTRODUCTION

The majority of human breast cancers overexpress c-Myc, leading to genomic instability, inappropriate cell cycle progression and seemingly paradoxically, to apoptosis (1-2). In the past, our laboratory and others have focused on uncovering the survival signaling pathways responsible for inhibiting c-Myc-mediated apoptosis, and thus contributing to tumorigenesis. One of the pathways that provided a strong survival signal in our model (Myc83 cells, a mouse mammary carcinoma cell line derived from MMTV-c-Myc tumor) was the PI3K/Akt pathway (3). The serine/threonine kinase, Akt is considered a central player controlling cellular survival, apoptosis, and oncogenesis. (4-6). In our model, EGF stimulation of Myc83 resulted in immediate activation of Akt, and also prevented apoptosis of these cells (3). Introduction of a constitutively activated Akt molecule into Myc83 cells protected them from apoptosis induced by PD153835, an EGFR-tyrosine kinase inhibitor (3).

In search of upstream regulators of Akt, our lab discovered that calcium and calmodulin, the universal calcium-sensing molecule, mediated activation of Akt and regulated survival of c-Myc-overexpressing cells (7). Specifically, EGF-mediated activation of Akt was diminished or abolished by the internal calcium chelator, BAPTA-AM, and the specific calmodulin inhibitor, W7 (7). Additionally Akt activation by serum and insulin was also abolished by calcium inhibition, suggesting that calcium may be a common regulator of Akt, irrespective of activating ligand. Additionally, calcium/calmodulin mediated Akt activation operates in non-tumorigenic human mammary epithelial cells independent of their overexpression of c-Myc (7). However, only c-Myc-overexpressing mouse mammary carcinoma cells undergo apoptosis in the presence of the calmodulin inhibitor W7, demonstrating the fundamental role of calmodulin in survival of these cells (7).

Calcium, a known intercellular signaling molecule, exerts many of its signalling properties by interacting with calmodulin, a calcium binding protein (8,9). Calmodulin, coupled with calcium, is able to interact with and activate a variety of kinases called calcium-calmodulin dependent protein kinases (CaM kinases). CaM Kinases are extensively studied in the nervous system where they play a critical role in intra-neuronal signaling and in muscular tissue where many of their substrates have been identified, including cytoskeletal protein such as actin and myosin (10). Their role in mammary epithelial cell signaling has only recently been investigated.

Recently, the role of certain calcium/calmodulin dependent protein kinases has been explored in a number of epithelial tissues. One CaM kinase, Pregnancy-upregulated nonubiquitous CaM kinase, Pnck, was recently discovered to be expressed in the developing mouse mammary gland, and was specifically upregulated in a subset of mammary epithelial cells during pregnancy (11-12). Pnck was also expressed in human breast tumors but not in adjacent benign tissues. Pnck mRNA was also detected in a subset of human breast cancer cell lines and, interesting, specifically upregulated in mouse mammary tumors resulting from MMTV-driven overexpression of c-Myc, but not from tumors derived from other MMTV-oncogene-driven transgenic mice (12). These observations suggest that Pnck may be associated with transformation of the mammary epithelium, and possibly in a c-Myc-associated manner. Interestingly, in cell culture experiments, Pnck mRNA was seen to be upregulated in growth-arrested cells, either by over confluent culture conditions or serum starvation (12). Additionally, Pnck's expression in the mammary gland during pregnancy was restricted to a subset of epithelial cells during late pregnancy when the mammary gland is undergoing differentiation. Pnck was

also seen in mammary epithelial cells undergoing post lactational involution, suggesting that Pnck's expression is correlated with differentiation and apoptosis of mammary epithelial cells (12).

As mentioned above, we have recently uncovered the survival mechanism(s) operating in Myc83 cells (mouse mammary carcinoma cells derived from MMTV-c-Myc tumors (7). In these cells, which overexpress calmodulin, a unique role of calcium and calmodulin was discovered. In Myc83 cells, ligand mediated activation of Akt was calcium/calmodulin dependent (7). Our lab hypothesized that Pnck expression may interact with this calcium/calmodulin-Akt pathway, possibly by opposing CaM-mediated activation of Akt. We aim to uncover the relationship between Pnck and Akt in EGF-mediated signaling.

Pnck is a novel, completely uncharacterized protein, and the role of CaM kinases in the mammary epithelial cell proliferation and transformation is virtually unexplored. Because of the lack of understanding of CaM kinases in breast cancer, and because of the potential therapeutic benefit of characterizing such a novel kinase, we proposed to clone the human Pnck gene and investigate its role in human breast cancer cells.

BODY:

STATEMENT OF WORK:

Hypothesis: We proposed that Pnck, the pregnancy upregulated non-ubiquitous CaM kinase, has anti-proliferative or pro-apoptotic effect on mammary epithelial cells during or after the transformation process.

Aim 1: Cloning of the human Pnck gene and functionally characterize its role in mammary carcinoma cells.

Aim 1a: Cloning of the human Pnck gene. [STATUS: Completed]

Aim 1b: Express Pnck in a transient and stable manner, as well as Pnck mutants in human mammary carcinoma cells compared to empty vector control [STATUS: ongoing]

Aim 1c: Functionally determine the effect of wild-type Pnck expression and Pnck mutants on cell proliferation and apoptosis in vitro. [Status: ongoing]

Aim 2: We aim to clone the Pnck promoter and determine the fundamental transcriptional regulatory elements controlling Pnck expression.

Aim 2a: Clone the Pnck promoter [Status: Not yet begun]

Aim 2b: Analyze the Pnck promoter to determine the basic transcriptional regulation of the human pnc gene. [Status: Not yet begun]

Aim 2c: Determine the relationship between c-myc and Pnck regulation. [Status: Not yet begun]

Progress Aim 1:

Hypothesis: We proposed that Pnck, the pregnancy upregulated nonubiquitous CaM kinase has an anti-proliferative or pro-apoptotic effect on mammary epithelial cells during or after the transformation process.

Aim 1: We aim to clone the human Pnck gene and functionally characterize its role in mammary carcinoma cells

Aim 1a: Cloning of the human Pnck gene. This aim was completed and described in the previous report.

Aim 1b: Express Pnck in a transient and stable manner, as well as Pnck mutants in human mammary carcinoma cells compared to empty vector control.

In the last annual report, we detailed the status of Pnck expression in a transient model, a constitutive stable MCF-7 and T47D model, and inducible MCF-7 Tet-on stable model. For the majority of experiments, we have focused on using transient expression of Pnck in a number of cell lines. Consistently, we have had difficulty detecting Pnck expression and a time course experiment evaluating the presence of Pnck in MCF-7 after transfection revealed that Pnck is degraded between 24-48 hours post transfection (See Figure 1).

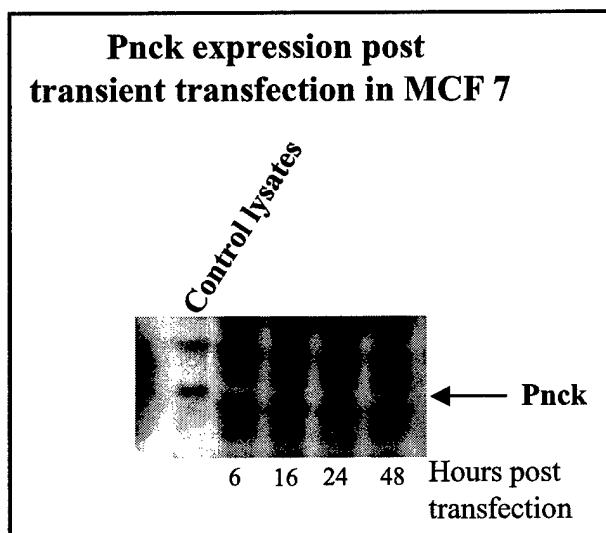


Figure 1. Exogenous expression of Pnck in MCF-7 cells is lost after 24 hours. Transient transfection of Pnck into MCF-7 cells results in expression of Pnck up to 24 hours post transfection. Pnck expression is lost in MCF-7 cells by 48 hours. MCF-7 cells were transfected with wild-type Pnck and grown in complete media. Pnck expression was detected in the lysates of MCF-7 cells using anti-Pnck polyclonal antibody.

In an attempt to rescue Pnck expression in a transient transfection model, we employed the use of proteasome inhibitors such as MG-132 to determine if proteosomal degradation was responsible for the rapid elimination of Pnck from the cell. Inhibition of the proteasome did not rescue Pnck expression (data not shown). We have begun cell fractionation experiment to see if Pnck is translocated to a different cellular compartment and unseen in cytosolic lysates. These experiments are ongoing and currently inconclusive.

In an attempt to express a constitutively activated, non-regulated construct, we developed an N-terminal HA-tagged Pnck construct to that we could create a C-terminal deleted Pnck construct that would eliminate Pnck's proposed C-terminal regulatory domain. The HA-tag at the N-terminal would allow for Pnck detection since removal of the C-terminus of Pnck would also eliminate our antibody recognition site.

The following constructs were generated:

- N-terminal HA-tagged wild-type Pnck
- N-terminal HA-tagged Pnck mutant K48M, ATP binding site mutant
- N-terminal HA-tagged Pnck mutant K49M, ATP binding site mutant
- N-terminal HA-tagged Pnck mutant K48/49M, ATP binding site mutant
- N-terminal HA-tagged Pnck mutant T171A, direct phosphorylation site mutant
- N-terminal HA-tagged Pnck mutant W296K, calmodulin binding site mutant
- N-terminal HA-tagged Pnck mutant Δ 271, a c-terminally truncated Pnck

These mutants will be used in an immuno-kinase assay, where Pnck and Pnck mutants can be expressed in eukaryotic cells, activated, and immunoprecipitated via HA-antibody and studied for direct activity against peptide targets. This will be studied under Aim 3, which seeks to determine Pnck's function in breast carcinoma cells.

Aim 3: Functionally determine the effect of Pnck expression and Pnck mutants on cell proliferation and apoptosis in vitro.

Investigation of Pnck's function in a transiently expressed Pnck model has proven difficult, possibly due to the rapid removal of Pnck expression in transfected cells. To overcome this, we are currently investigating the effect of Pnck elimination via siRNA experiments in Pnck expressing breast cancer cells. These experiments are ongoing.

Results of functional experiments in transiently transfected Pnck cells were generally negative. To study the effect of Pnck on cell cycle we cotransfected an enhanced green fluorescent protein (eGFP) construct with Pnck and selected only eGFP expressing cells for FACS analysis. Consistently, both Pnck transfected and vector control transfected cells displayed similar cell cycle profiles. Optimization of time and culture conditions did not reveal an effect of Pnck on cell cycle by FACS analysis (data not shown). Additionally, early passage, stable MCF-7-Pnck cells and MCF-7-control cells were indistinguishable in regards to their cell cycle profiles (data not shown). Consistent with these results, proliferation assays performed on the MCF-7 stable cells revealed identical growth curves, suggesting that Pnck does not have an effect on cell proliferation (data not shown).

To investigate the mode of action of Pnck in mammary epithelial cells we decided to use our transient transfection model to examine the biochemical effect of Pnck on known survival pathways in breast cancer cell lines. We had previously revealed that mammary epithelial and carcinoma cells employ calcium/calmodulin to mediate the EGF-induced activation of Akt. We sought to determine if Pnck, a CaM kinase, was exerting an effect in this pathway.

In MCF-7 cells, wild-type, untagged Pnck or empty vector was cotransfected with an HA-tagged Akt molecule. Cells were recovered from transfection and serum starved for three hours before stimulation with various ligands. Stimulated cells were lysed and exogenous HA-Akt was immunoprecipitated from the lysates using an HA-antibody and captured on Sepharose G beads. Immunoprecipitates were run on a gel and Akt activation was examined using P-Akt antibodies (S473 and T308). In MCF-7 cells, we observed that Pnck enhanced Akt activation upon ligand stimulation (see Figure 2).

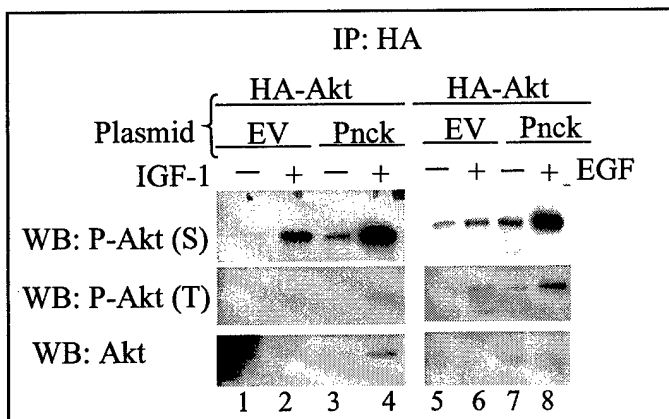


Figure 2. Ectopic Pnck expression augments IGF-I/EGF-induced Akt activation in MCF-7 cells. MCF-7 cells were transiently co-transfected with empty vector (EV) and HA-Akt (lanes 1-2; lanes 5-6) or Pnck construct and HA-Akt (lanes 3-4; lanes 7-8). Cells were serum starved and stimulated with or without IGF-I (lanes 1-4) or EGF (lanes 5-8), lysed and HA-Akt was immunoprecipitated by anti-HA mAb

(12CA5). Immunoprecipitates were probed for activation specific (S-473P/T-308P) Akt antibodies.

To determine if the effect of Pnck on Akt activation was direct we performed a series of immuno-kinase assays where N-terminally HA-tagged Pnck was transfected into MCF-7 and HEK293T cells. After recovery, cells were serum starved and stimulated by EGF, IGF and insulin. HA-Pnck was immunoprecipitated by HA-antibody and incubated in a kinase reaction mixture containing γ -ATP and 300 μ M of peptide target. To determine if Pnck was directly phosphorylating Akt, peptides of wild-type Akt S473 site and mutated Akt S473A were generated and used as targets in the HA-Pnck kinase assay. Consistently and reproducibly, Pnck did not demonstrate kinase activity against the Akt peptide targets (data not shown). This suggests that Pnck-enhanced activation of Akt is most likely indirect. Preliminary results suggest that Pnck may be upregulating Akt expressing or controlling Akt translation and we are currently investigating this.

Progress Aim 2:

Work on Aim 2 has not begun. Promoter analysis of Pnck will be initiated after the biochemical and functional investigation of Pnck are complete.

Aim 2: We aim to clone the PNCK promoter and determine the fundamental transcriptional regulatory elements controlling PNCK expression.

Aim 2a: Clone the PNCK promoter:

Aim 2b: Promoter analysis to determine basic transcriptional regulation of the human PNCK gene.

Aim 2c: Determine the relationship between c-Myc and PNCK regulation.

Progress on Aim 2:

To date, Aim 2 has not been started; we leave aim 2 unchanged.

Aim #2: Year 2: As previously expressed in the revised statement of work:

We aim to clone the PNCK promoter and determine the fundamental transcriptional regulatory elements controlling PNCK expression.

Aim 2a: Clone the PNCK promoter:

In order to understand the basic transcriptional regulation of the human PNCK, we propose cloning of the PNCK promoter. We aim to do this by cloning the PNCK promoter from a human genomic library by using the PCR-based Promoter Finder DNA Walking kit (Promega). Gene specific primers will be derived from the 5'UTR of the human PNCK gene. The full length promoter will be cloned and sequenced. The transcription start site of the human gene will be determined using primer extension analysis with nested primers derived from known DNA sequence.

Aim 2b: Promoter analysis to determine basic transcriptional regulation of the human PNCK gene.

First, sequence analysis of the promoter will be performed to determine the presence of consensus transcription factor binding sites present in the human PNCK promoter sequence. To identify the functional promoter elements involved in PNCK gene regulation, progressive 5' deletion mutants will be constructed based on the location of consensus factor binding sites on the promoter. These promoter mutants as well as the wild-type promoter will be cloned into a luciferase vector, transfected into human breast cancer cells and their relative luciferase activity will be assayed. In addition to the 5' deletion mutations, internal mutation and deletions will be made by PCR based site-directed mutagenesis, based on the results of the 5' deletions. These internal deletions and mutations will be cloned into the luciferase vector for transfection into human breast cancer cells.

Aim 2c: Determine the relationship between c-Myc and PNCK regulation.

Since MMTV-c-myc but not MMTV-ras mice demonstrated a specific upregulation of PNCK in their mammary tumors, c-Myc may be a direct transcriptional regulator of PNCK (16). Using unique, stable c-Myc expressing mammary epithelial cell line created in our laboratory, (MCF10A-Myc) and a 4-OHT regulatable Myc, Myc-ER (MCF10A-MycER) we can determine if c-Myc activity results in PNCK expression and directly investigate the relationship between c-Myc and PNCK regulation (13).

KEY RESEARCH ACCOMPLISHMENTS

-Uncovered that transient Pnck expression is diminished after 24 hours post transfection and lost by 48 hours post transfection. We have determined that loss of Pnck expression is most likely not mediated by degradation by the proteasome.

-Development of an N-terminally tagged wild type Pnck construct and HA-tagged Pnck mutations in the APT binding site, phosphorylation site, and calmodulin binding site, as well as a C-terminally truncated Pnck mutant that removed the putative regulatory domain of Pnck.

-We have determined that Pnck does not affect the cell cycle of breast cancer cells, and does not affect proliferation of breast cancer cells.

-Pnck enhances EGF and IGF induced Akt-activation in MCF-7 cells.

- We have developed an Immuno-kinase assay for Pnck.
- Immuno-kinase assay of Pnck revealed that Pnck is not directly phosphorylating Akt S473.
- Pnck-enhanced Akt phosphorylation is not direct.
- Pnck may affect ligand-mediated Akt phosphorylation by enhancing Akt transcription or translation.

REPORTABLE OUTCOMES

-Co-authorship of a Journal of Biological Chemistry publication (see APENDIX):
 Deb, T.B., Coticchia, C.M., Dickson, R.B. Calmodulin-mediated activation of Akt regulates survival of c-Myc-overexpressing mouse mammary carcinoma cells. (2004) Journal of Biological Chemistry 279 (37):38903-11.

CONCLUSIONS

Here we aim to characterize a novel CaM kinase, Pnck, in its function, regulation, and biochemical activity. Generation of an adequate model system to study Pnck's biological function has proved difficult, partly because of Pnck rapid loss of expression after transient transfection. This expression profile may have contributed to our past difficulties in development of both inducible stable expressing Pnck cell lines as well as constitutive expressing Pnck cell lines. We have optimized a transient transfection model to study Pnck's biochemical function and have designed functional studies around these expression constraints. We have determined that Pnck does not affect the cell cycle or cell proliferation in either a positive or negative manner. We have observed that Pnck enhances ligand mediated Akt activation and are in the process of uncovering the mechanism of this action. Simultaneously, we will determine if Pnck-enhanced Akt activation provides an anti-apoptotic, survival advantage to cells exposed to chemotherapeutic agents and apoptotic ligands, such as FasL. The observation that Pnck enhances Akt activation is opposite from our expected observation that Pnck may be an anti-proliferative, or pro-apoptotic molecule, but this is not unusual considering that nothing was known about Pnck other than its expression profile before these experiments were initiated. We look forward to further characterizing Pnck's biological function, biochemical profile and regulation.

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APPENDIX

Deb, T.B., Coticchia, C.M., Dickson, R.B. Calmodulin-mediated activation of Akt regulates survival of c-Myc-overexpressing mouse mammary carcinoma cells. (2004) *Journal of Biological Chemistry* **279** (37):38903-11.

Calmodulin-mediated Activation of Akt Regulates Survival of c-Myc-overexpressing Mouse Mammary Carcinoma Cells*

Received for publication, May 12, 2004, and in revised form, July 7, 2004
Published, JBC Papers in Press, July 9, 2004, DOI 10.1074/jbc.M405314200

Tushar B. Deb, Christine M. Coticchia‡, and Robert B. Dickson§

From the Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, D. C. 20057

c-Myc-overexpressing mammary epithelial cells are proapoptotic; their survival is strongly promoted by epidermal growth factor (EGF). We now demonstrate that EGF-induced Akt activation and survival in transgenic mouse mammary tumor virus-c-Myc mouse mammary carcinoma cells are both calcium/calmodulin-dependent. Akt activation is abolished by the phospholipase C- γ inhibitor U-73122, by the intracellular calcium chelator BAPTA-AM, and by the specific calmodulin antagonist W-7. These results implicate calcium/calmodulin in the activation of Akt in these cells. In addition, Akt activation by serum and insulin is also inhibited by W-7. EGF-induced and calcium/calmodulin-mediated Akt activation occurs in both tumorigenic and non-tumorigenic mouse and human mammary epithelial cells, independent of their overexpression of c-Myc. These results imply that calcium/calmodulin may be a common regulator of Akt activation, irrespective of upstream receptor activator, mammalian species, and transformation status in mammary epithelial cells. However, only c-Myc-overexpressing mouse mammary carcinoma cells (but not normal mouse mammary epithelial cells) undergo apoptosis in the presence of the calmodulin antagonist W-7, indicating the vital selective role of calmodulin for survival of these cells. Calcium/calmodulin-regulated Akt activation is mediated directly by neither calmodulin kinases nor phosphatidylinositol 3-kinase (PI-3 kinase). Pharmacological inhibitors of calmodulin kinase kinase and calmodulin kinases II and III do not inhibit EGF-induced Akt activation, and calmodulin antagonist W-7 does not inhibit phosphotyrosine-associated PI-3 kinase activation. Akt is, however, co-immunoprecipitated with calmodulin in an EGF-dependent manner, which is inhibited by calmodulin antagonist W-7. We conclude that calmodulin may serve a vital regulatory function to direct the localization of Akt to the plasma membrane for its activation by PI-3 kinase.

One of the fundamental etiologic processes in tumorigenesis is the ability of cancer cells to evade programmed cell death, or apoptosis (1). Potent cell survival signaling, in parallel with

uncontrolled cell proliferation and other processes, ultimately leads to the development of a malignant tumor. In breast cancer, this pathologic outcome is strongly influenced by growth factors and/or hormones, which interact with their cognate receptors on mammary epithelial cells. Receptor-ligand interactions at the cell surface are propagated as cascades of signals through the cytoplasm, culminating in specific gene expression programs in the nucleus to define specific biological outcome(s). The serine/threonine kinase Akt is considered a central player controlling cellular survival (2), apoptosis (3), and oncogenesis (4, 5). Akt is activated by growth factors and other stimuli, through both phosphatidylinositol 3-kinase (PI-3 kinase)¹-dependent and independent mechanisms (6–9). PI-3 kinase, a ubiquitous lipid kinase and upstream effector of Akt (10), has also been implicated in a variety of cellular functions, including survival and antiapoptosis (11, 12), growth and proliferation (13, 14), differentiation (15, 16), cytoskeletal rearrangement (17), translocation of glucose transporter GLUT4 (18, 19) and membrane ruffling (20). Upon growth factor stimulation, PI-3 kinase generates 3'-phosphorylated phosphoinositides, such as phosphatidylinositol 3,4-bisphosphates and phosphatidyl inositol 3,4,5-trisphosphates, at the plasma membrane. These phosphoinositides serve as binding anchors for the Pleckstrin homology domain of Akt and thus encourage translocation of Akt to the plasma membrane (10, 21–23). At the plasma membrane, Akt is phosphorylated at Ser-473 and Thr-308 and fully activated (24). In addition to a PI-3 kinase-dependent mechanism of Akt activation, a PI-3 kinase-independent mechanism(s) of Akt activation has also been reported. The prime candidate mediating this mechanism is calmodulin kinase kinase, which directly phosphorylates Akt in a calcium-dependent manner (6). Calmodulin, the allosteric regulator of calmodulin kinases, also regulates PI-3 kinase-dependent Akt activation, independent of calmodulin kinase kinase, and is known to control neuronal cell survival (25, 26) and GLUT4 translocation in 3T3-L1 adipocytes (27). Calmodulin binds to the p85 α regulatory subunit of PI-3 kinase (28), but this binding does not result in the generation of phosphatidyl inositol 3,4,5-phosphates, which are required for membrane targeting of Akt and for its subsequent activation (10). A calcium/calmodulin-dependent PI-3 kinase (hVPS34) cascade, responsible for phagosome maturation, has recently been reported (29). A consensus sequence in the p110 catalytic subunit of PI-3 kinase has been predicted to be the binding site of calmodulin, but no biochemical data currently exist to support this idea (30). Be-

* This work was supported in part by National Institutes of Health Grant R01-CA72460 and United States Department of Defense Grant DAMD 17-01-1-0251 (to R. B. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a predoctoral training grant from the United States Department of Defense (DAMD 17-01-1-0246).

§ To whom correspondence should be addressed: Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, New Research Building, Room W417, 3970 Reservoir Road NW, Washington, D.C. 20057. Tel.: 202-687-3770; Fax: 202-687-7505; E-mail: dicksonr@georgetown.edu.

¹ The abbreviations used are: PI-3 kinase, phosphatidylinositol 3-kinase; MMTV, mouse mammary tumor virus; FBS, fetal bovine serum; EGF, epidermal growth factor; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid-acetoxymethyl ester; MAP, mitogen-activated protein; pAb, polyclonal antibody; PARP, poly(ADP-ribose) polymerase; PI, phosphatidylinositol; PKC, protein kinase C; EF-2, elongation factor 2; PLC, phospholipase C; EGFR, epidermal growth factor receptor.

cause mammary epithelial cells can release abundant calcium from intracellular stores in response to growth factors and survival ligands (31, 32), we hypothesized that calcium plays a major role in survival of these cells. Our laboratory has already reported that EGF-induced survival of c-Myc-overexpressing mammary carcinoma cells is mediated by activation of PI-3 kinase/Akt kinase (33). In search of a specific survival mechanism downstream of EGFR, which may be a therapeutic target in breast carcinoma, and to uncover any existing relationship between PI-3 kinase and calcium mediated activation of Akt, we undertook further investigation of calcium- and PKC-dependent survival mechanism(s). Our new studies resulted in the identification of a calcium/calmodulin-dependent Akt activation and survival mechanism in these cells. In particular, EGF-induced Akt activation is mediated by calmodulin, the universal calcium sensor, resulting in cell survival. We have shown that calmodulin does not exert its effect directly at the PI-3 kinase level. We have further shown that an EGF-dependent complex forms between calmodulin and Akt. This mechanism probably transports Akt to the plasma membrane for its activation by a PI-3 kinase-dependent mechanism. Perturbation of this targeting mechanism by calmodulin antagonism leads to apoptotic cell death in tumorigenic mammary carcinoma cells. This novel mechanism may have broader implications in the regulation of breast cancer, GLUT4 translocation, and neuronal survival.

EXPERIMENTAL PROCEDURES

Cell Lines—Mammary tumor-bearing MMTV-c-Myc transgenic and MMTV-c-Myc/MT-TGF α bitransgenic mice have been described previously (34–36). Mammary tumor-derived carcinoma cells from these mice were cultured in improved modified Eagle's minimum essential medium containing 2.5% fetal bovine serum (FBS), 10 ng/ml EGF, and 5 μ g/ml insulin. The MMTV-c-Myc transgenic tumor cell lines Myc83, Myc9, and Myc7 were examined, and representative experiments were performed in Myc83 cell lines. Likewise, Myc975 was chosen as a representative cell line of MMTV-c-Myc/MT-TGF α bitransgenic tumors. The Comma D cell line was previously derived from normal mammary epithelium (37–38); cells were cultured in improved modified Eagle's minimum essential medium containing 5% FBS. Isolation and maintenance of non-tumorigenic immortal human mammary epithelial cell lines 184A1N4 and 184A1N4-Myc were described previously (39, 40). A description of retroviral transfection, selection, and development of immortalized MCF 10A-LXSN and MCF 10A-c-Myc stable human mammary epithelial cell lines was published previously (41).

Antibodies and Reagents—Pharmacological inhibitors staurosporine, BAPTA-AM, GF109203X, W-12, W-7, KN-62, KN-92, AG1478, and Rotlerin were purchased from Calbiochem. STO-609 was from Tocris Cookson Inc. Phospho-Akt (Ser-473), Phospho-Akt (Thr-308), phospho-p44/42 MAP kinase (Thr-202/Tyr-204) and phospho-EF2 (Thr-56) and Akt pAb were obtained from Cell Signaling Technology, Inc. Akt 1(C-20) goat polyclonal Ab, PARP pAb (H-250), and CaM I (FL-149) pAb were purchased from Santa Cruz Biotechnology, Inc. U-73122, N-benzoyloxycarbonyl-VAD-fluoromethyl ketone, and immobilized bovine calmodulin on Sepharose were purchased from BIOMOL Research Laboratories. α -Tubulin, Ab-2 monoclonal antibody was from NeoMarkers, Inc. Pan-extracellular signal-regulated kinase monoclonal antibody was obtained from BD Biosciences. Monoclonal calmodulin antibody, p85 α pAb, anti-phosphotyrosine monoclonal antibody (clone 4G10), and biotin-conjugated anti-phosphotyrosine (clone 4G10) were purchased from Upstate Biotechnology. Hoechst stain was from Sigma. Phosphatidylinositol was purchased from Avanti Polar Lipids.

Treatment with Pharmacological Inhibitors and Preparation of Whole Cell Lysates—Semiconfluent cell monolayers were serum-starved overnight and then incubated with inhibitors for indicated periods of time. Cells were stimulated with 10 nM EGF for 3 min or for the indicated time periods (as shown in the text and figure legends) (in time course experiments) at 37 °C, and lysed in lysis buffer (10 mM Tris-base, pH 7.4, 1% Triton-X-100, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM β -glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, and 2 μ g/ml each of pepstatin, leupeptin, and aprotinin). Lysates were vortexed and centrifuged at 15,000 \times g for 15 min at 4 °C.

Protein concentrations of lysates were measured using BCA protein assay kit (Pierce) and Ultramark Microplate Imaging System (Bio-Rad).

Immunoprecipitation and Western Blotting—Immunoprecipitations and Western blotting were performed as described previously (42). In brief, 1 μ g of antibody was added to 500 μ g of clarified whole cell lysates and incubated for 1 h at 4 °C. 5 μ l of protein A-agarose beads were added, and lysates were further incubated for 1 h at 4 °C. Beads were precipitated by centrifugation at 15,000 \times g for 2 min and washed 3 times in lysis buffer. Bound proteins were released by boiling in SDS-PAGE sample buffer for 3 min. Proteins were resolved on SDS-PAGE and transferred to polyvinylidene difluoride (Immobilon-P; Millipore) membranes. Membranes were incubated in primary antibody for 2 h, followed by biotinylated secondary antibody for 1 h, and detected by Vectastain ABC Elite kit (Vector Laboratories) and enhanced chemiluminescence (PerkinElmer Life and Analytical Sciences).

Hoechst Staining and PARP Cleavage Assays—Semiconfluent growing cells were transferred to complete medium containing either 30 μ M W-12 or W-7. After 36 h, all floating and adherent cells were collected. Samples were centrifuged for 8 min at 1000 \times g at 4 °C. Supernatants were discarded, and cell pellets were suspended in a 1 \times phosphate-buffered saline solution containing 0.3% formaldehyde and 2% Nonidet P-40 and stained with 10 μ g/ml of Hoechst 33258 dye (Sigma) for apoptotic analysis. For each replicate, at least 500 cells were counted and evaluated for the presence of condensed nuclei and overall apoptotic appearance. For PARP cleavage assays, a third plate was treated with W-7, in the presence of 50 μ M N-benzoyloxycarbonyl-VAD-fluoromethyl ketone, a broad-spectrum caspase inhibitor. After 16 h, adherent cells were trypsinized and lysed, and equal amounts of total proteins were resolved in SDS-PAGE and immunoblotted by anti-PARP antibody.

PI-3 Kinase Assay—PI-3 kinase assays were performed, using a modified protocol from Kapeller *et al.* (44). In brief, anti-phosphotyrosine and anti-p85 α immunoprecipitates were washed twice in lysis buffer (phosphate-buffered saline, pH 7.5, 1% Nonidet P-40, and 100 μ M vanadate), twice in a second buffer (10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 100 μ M LiCl, and 100 μ M vanadate), and finally twice in a third buffer (10 mM Tris-Cl, pH 7.5, 100 mM NaCl, and 100 μ M vanadate). Lipid kinase assays were performed on washed beads at 37 °C for 10 min in a reaction mixture containing 20 mM HEPES, pH 7, 40 μ M ATP, 10 mM MgCl₂, 100 μ M vanadate, 0.2 μ g/ μ l sonicated phosphatidylinositol (PI), and 20 μ Ci of [γ -³²P]ATP (6000 μ Ci/mmol). At the end of the reaction, 80 μ l of HCl (1 M) were added, followed by 160 μ l of methanol/chloroform (1:1), and the organic layer was extracted and spotted on oxalate/EDTA-pretreated thin layer chromatography plates. Thin layer chromatography plates were resolved using a running buffer containing 60:40:11.3:2 chloroform/methanol/H₂O/ammonium hydroxide.

RESULTS

EGF-induced Akt Activation Is Inhibited by Staurosporine but Not by GF109203X—Our previous observations implicated EGF-induced PI-3 kinase/Akt activation as a survival mechanism of MMTV-c-Myc mouse mammary carcinoma cells (henceforth called Myc83 cells) (33). We next wanted to investigate the possible upstream signaling mechanisms regulating this Akt kinase activation and cell survival. In a previous study with small cell lung cancer cells, c-Myc sensitized these cells to apoptosis during nutrient depletion (45). Under these conditions, PKC- β 2 overexpression improved cell survival by protecting against c-Myc-induced apoptosis. PKC δ is also known to promote survival of small cell lung cancer cells (46). By analogy, we hypothesized that EGF-induced PKC activation, upstream of PI-3 kinase, might be responsible for Akt activation. To begin to test this idea, serum-starved Myc83 cells were incubated with staurosporine, a broad spectrum PKC inhibitor, as well as GF109203X, a specific inhibitor of PKC (all isoforms) (47–49), for 30 min, followed by stimulation with 10 nM EGF for 3 min. Cells were lysed, and equal amounts of total protein-containing lysates were probed by activation specific anti-phospho Akt (Ser-473) antibody. Although 1 μ M staurosporine completely inhibited EGF-induced Akt activation, 5 μ M GF109203X had no effect (Fig. 1A, WB: P-Akt S-473). 5 μ M GF109203X also failed to inhibit EGF-induced Akt activation in serum-starved, bitransgenic MMTV-c-Myc/MT-TGF α mouse

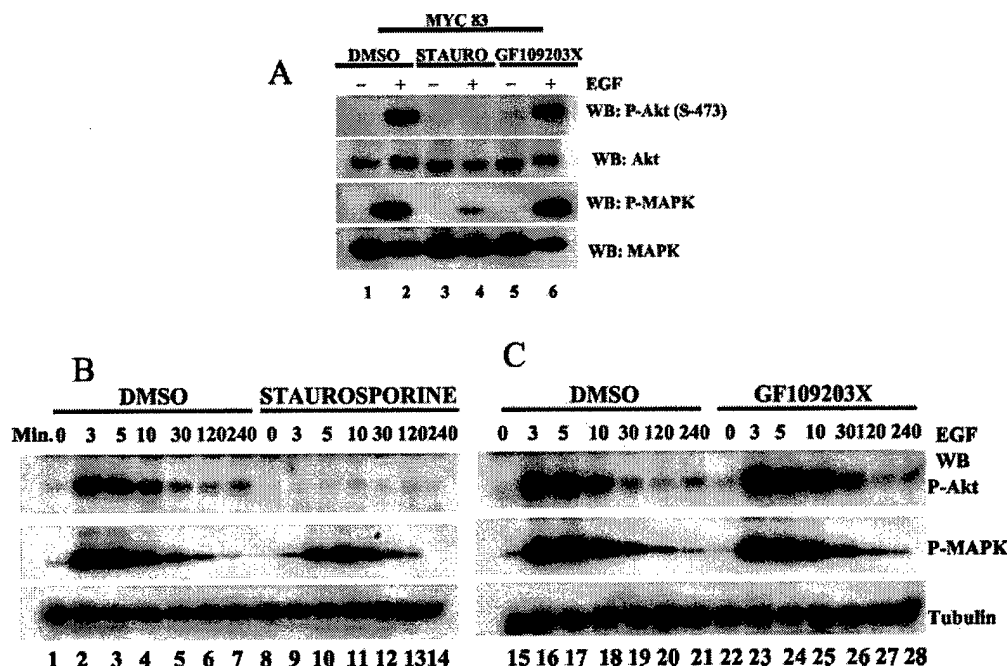


FIG. 1. Effect of staurosporine and GF109203X on EGF-induced Akt activation. A, staurosporine, but not GF109203X, inhibits EGF-induced Akt activation in Myc83 cells. Serum-starved cells were preincubated with Me₂SO (lanes 1 and 2), 1 μ M staurosporine (lanes 3 and 4), and 5 μ M GF 109203X (lanes 5 and 6) for 30 min and treated with or without 10 nM EGF for 3 min. Equal amounts of total protein containing cell lysates from each sample were resolved on SDS-PAGE and transferred to polyvinylidene difluoride membrane. Membrane was probed by anti-phospho Akt (Ser-473) antibody [WB: P-Akt (S-473)] or anti-phospho-MAP kinase antibody (WB: P-MAPK). Each blot was reprobed by anti-Akt (WB: Akt) and anti-MAP kinase antibody (WB: MAPK), respectively. B and C, time course experiment reveals sustained Akt inhibition but transient MAP kinase inhibition by staurosporine but not by GF109203X. Serum-starved cells were incubated with Me₂SO (DMSO) and 1 μ M staurosporine (B) or 5 μ M GF109203X (C) and stimulated with 10 nM EGF for indicated time period. Lysates were probed for Akt (WB: P-Akt) and MAP kinase activation (WB: P-MAPK) as described in A. Each blot was reprobed by anti-tubulin antibody (WB: Tubulin) for consistent loading.

mammary carcinoma cells (henceforth called Myc α 75 cells) and non-malignant mouse Comma D cells (37–38) (data not shown). Staurosporine also caused significant inhibition of MAP kinase. However, GF109203X had no effect on MAP kinase activity (Fig. 1A, WB: P-MAPK). 5 μ M GF109203X inhibited PMA-induced MAP kinase activation in all of these cell lines, indicating that it is active in these cells at this concentration (data not shown). To examine whether GF109203X inhibits Akt activation at later time points, the effect of both staurosporine and GF109203X on Akt and MAP kinase activation were examined in a time course experiment. Staurosporine inhibited Akt activation in a sustained manner, whereas GF109203X did not inhibit Akt activity at any time point tested (Fig. 1, B and C, WB: P-Akt). On the other hand, MAP kinase activity was significantly inhibited by staurosporine at 3 min, but substantial activity was regained at later time points (Fig. 1B, WB: P-MAPK). Similar to results in the short term experiment, neither EGF-induced Akt activation nor MAP kinase activity was affected by GF109203X in the time course experiment (Fig. 1C, WB: P-Akt and WB: P-MAPK). Taken together, these data show that EGF-induced Akt activation is mediated by a staurosporine inhibitable-factor but not by PKC.

Calcium Chelator BAPTA-AM and Calmodulin Antagonist W-7 Inhibit EGF-induced Akt Activation—Staurosporine has been reported to inhibit a variety of kinases, including calcium/calmodulin kinase II (50) and PKC (51). Considering that mammary epithelial cells release abundant calcium from intracellular stores in response to EGF (31, 32), we hypothesized that a calcium-regulated signaling mechanism downstream of EGFR might be a potential effector of Akt activation. To test this idea, serum-starved cells were preincubated for 90 min with 10 μ M BAPTA-AM, an intracellular calcium chelator (52–54), and stimulated with EGF. BAPTA-AM completely inhibited Akt activity and partially inhibited EGF-induced MAP

kinase activity (Fig. 2A, WB: P-Akt S-473 and WB: P-MAPK), whereas incubation of the cells with 2 mM EGTA (external calcium chelator) affected activation of neither Akt nor MAP kinase (Fig. 2A, WB: P-Akt S-473 and WB: P-MAPK). Identical results were obtained in both Myc α 75 and Comma D cells (data not shown). These results suggest that EGF-induced release of calcium from intracellular stores is required for Akt activation in Myc83, Myc α 75, and Comma D cells.

To further examine whether calcium mediates its effect on Akt activation through the universal calcium sensor calmodulin, we used a selective calmodulin antagonist, W-7 (27, 55). Calmodulin antagonists have been used previously for inhibiting nerve growth factor- and brain-derived neurotrophic factor-induced Akt activation, resulting in neuronal cell survival (25, 26). As shown in Fig. 2B, 30-min pretreatment of 30 μ M W-7, but not its inactive analogue W-12 (56), significantly inhibited EGF-stimulated Akt activation in Myc83 cells (WB: P-Akt S-473). Similar to W-7-treated Myc83 cells, Myc α 75 and Comma D cells also showed significant inhibition of their EGF-induced Akt activity (Fig. 3, B and C, WB: P-Akt S-473). The specificity of W-7 was confirmed by its ability to block EGF-induced dephosphorylation of elongation factor 2 (EF-2) (Fig. 3, A–C, WB: P-EF-2). EF-2 remains highly phosphorylated (Thr-56) in quiescent cells, thus inhibiting peptide chain elongation (57) and protein synthesis. Growth factors, such as insulin, cause dephosphorylation of EF-2 *via* calcium/calmodulin-dependent activation of calmodulin kinase III (formerly known as EF-2 kinase), resulting in peptide chain elongation. The calmodulin antagonist W-7 and the EF-2 kinase inhibitor Rotenone both effectively inhibit this dephosphorylation. Calmodulin has been implicated in survival of neuronal cells (25, 26) and chicken lymphoma B cells (58), but we are unaware of any report indicating a role for calmodulin in mediating Akt activation linked to mammary epithelial cell survival. Although

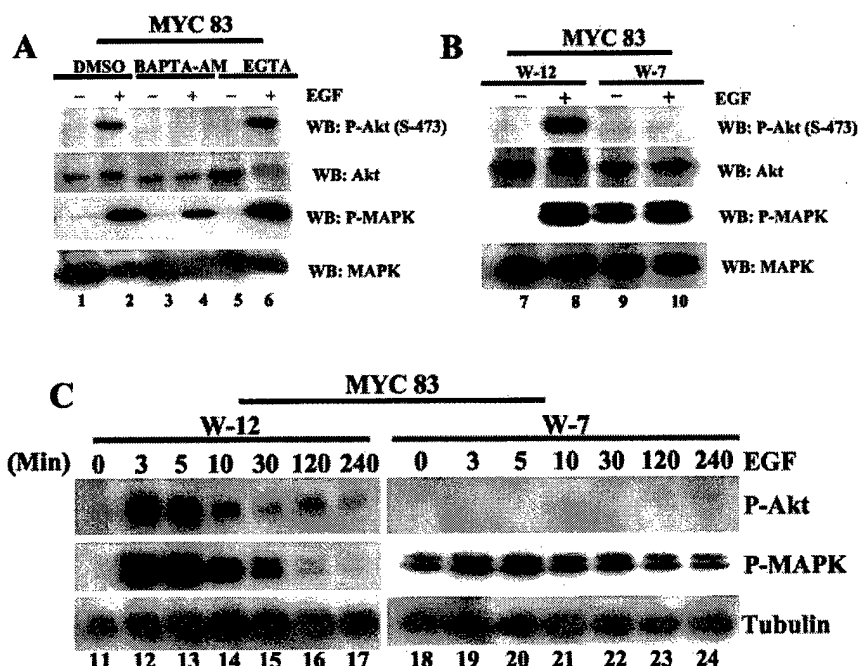


FIG. 2. Effect of intracellular calcium chelator, BAPTA-AM, and calmodulin antagonist W-7 on Akt activation in Myc83 cells. A, intracellular calcium chelator BAPTA-AM inhibits EGF-induced Akt activation. Myc83 cells were serum-starved overnight and incubated with Me_2SO (DMSO; lanes 1 and 2), 10 μM BAPTA-AM for 90 min (lanes 3 and 4), and 2 mM EGTA (lanes 5 and 6) for 90 min followed by 3 min of EGF stimulation. Lysates were probed for activated Akt [WB: P-Akt (S-473)] and activated MAP kinase (WB: P-MAPK). Blots were reprobed for total Akt (WB: Akt) and total MAP kinase (WB: MAPK), respectively. B, calmodulin antagonist W-7 inhibits Akt activation but up-regulates MAP kinase activation. Serum-starved cells were incubated with 30 μM W-7 (lanes 9 and 10) or inactive analogue W-12 (lanes 7 and 8) for 30 min. Cells were stimulated with 10 nM EGF for 3 min and processed for Akt and MAP kinase activation as described in Fig. 1A. C, sustained Akt inhibition and MAP kinase activation by calmodulin antagonist. Serum-starved Myc83 cells were preincubated with 30 μM W-7 (lanes 18–24) or inactive analogue W-12 (lanes 11–17) for 30 min, and stimulated with 10 nM EGF for indicated time period. Akt and MAP kinase activation were examined as described in Fig. 1B. Blots were reprobed by anti-tubulin antibody (WB: Tubulin) for consistent gel loading.

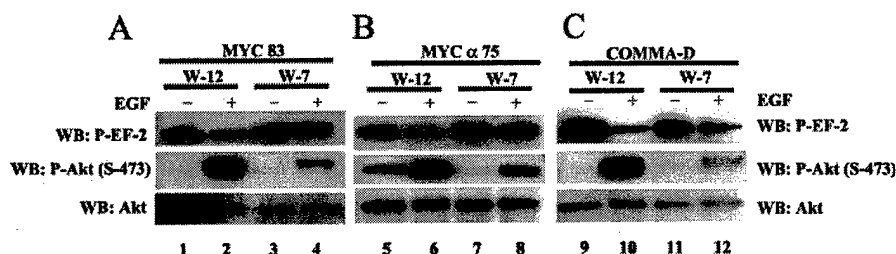


FIG. 3. Inhibition of Akt activation by calmodulin antagonist occurs in both tumorigenic carcinoma and non-tumorigenic mouse mammary epithelial cells. Tumorigenic cell lines Myc83 (lanes 1–4), Myc83 (lanes 5–8), and non-tumorigenic cell lines Comma D (lanes 9–12) were pre-incubated with either W-7 or W-12 for 30 min, stimulated with 10 nM EGF for 3 min, and processed for Akt activity exactly as described in Fig. 2B. An identical blot from each cell line was probed by anti-P-EF-2 (Thr-56) antibody (WB: P-EF-2).

calmodulin was shown to function upstream of Akt kinase leading to neuronal cell survival, the intermediate signaling mechanism is not completely understood.

We next examined the ability of calcium/calmodulin to activate Akt in a series of MMTV-c-Myc and MMTV-c-Myc/MT-TGF α transgenic mouse mammary tumor-derived cell lines, in addition to Myc83 and Myc83, respectively. In all cases, W-7 but not W-12 (inactive analogue) inhibited activation of Akt, thus excluding any effect of clonal variation in this mechanism (data not shown). However, W-7 incubation did not inhibit EGF-induced MAP kinase activity. It is interesting that the basal level of activated MAP kinase, as detected by anti-phospho-MAP kinase immunoblotting, increased in W-7-treated Myc83 cells (Fig. 2B, WB: P-MAPK). This is in agreement with previous observations of down-regulation of Ras/Raf/ERK pathway by calmodulin and of activation of MAP kinase activity by calmodulin antagonists, observed in NIH 3T3 fibroblasts (43, 59)

To examine whether the effect of W-7 on Akt or MAP kinase activation is sustained, we preincubated Myc83 cells with 30 μM W-7 and then stimulated them with EGF for different time periods. Anti-phospho-Akt (S-473) immunoblotting of the lysates revealed sustained inhibition of Akt activity by W-7, compared with inactive analogue, W-12 (Fig. 2C, WB: P-Akt). In contrast, W-7 incubation resulted in a sustained MAP kinase activation (Fig. 2C, WB: P-MAPK). Sustained MAP kinase activation is linked to both proliferation and differentiation, depending upon the cell line. In PC-12 cells, nerve growth factor-mediated, sustained MAP kinase activation results in differentiation (60). In contrast, sustained calmodulin inhibition, in serum-starved fibroblasts cells, induces extracellular signal-regulated kinase 2 phosphorylation and p21^{cip1} expression, leading to inhibition of cellular proliferation (59). Taken together, our data suggest that EGF-induced activation of Akt is mediated by calcium/calmodulin-dependent mechanism(s) and that calmodulin has opposing effects on sustained Akt and

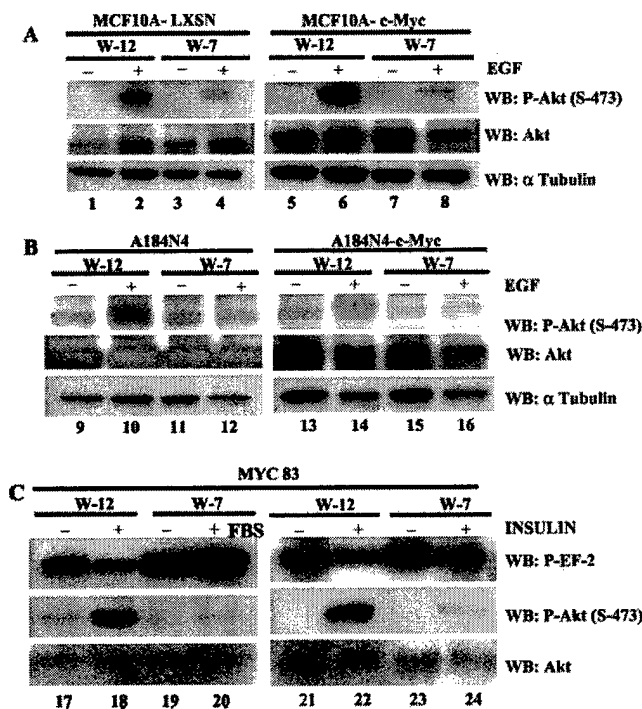


FIG. 4. Calmodulin antagonism inhibits EGF-induced Akt activation in both c-Myc-overexpressing and normal human mammary epithelial cell lines. Calmodulin antagonism also inhibits FBS and insulin-induced Akt activation. Human MCF-10A-LXSN (lanes 1–4) and MCF-10A-c-Myc (lanes 5–8) (A), human A184N4 (lanes 9–12) and A184N4-c-Myc (lanes 13–16) cells (B), and mouse Myc83 cells (C) were serum-starved and incubated with 30 μ M W-7 or W-12 for 30 min and stimulated either with or without 10 nM EGF for 3 min (A and B), 10% FBS for 5 min (lanes 17–20), 100 nM insulin for 5 min (lanes 21–24) (C). Lysed cells were examined for Akt (WB: P-Akt S-473) or EF-2 phosphorylation (WB: P-EF2) and total Akt (WB: Akt) as described previously.

MAP kinase activation in Myc83 cells.

Calmodulin Is a Common Central Regulator of Akt Activation, Irrespective of Ligands, Species, Tumorigenicity, and c-Myc Expression Status.—To investigate whether calmodulin-mediated activation of Akt occurs in other mammary epithelial cell systems, we tested the c-Myc-overexpressing, non-tumorigenic human mammary epithelial cell lines 184A1N4-Myc (39, 40) and MCF10A-c-Myc (41), along with their control counterparts 184A1N4 and MCF10A-LXSN. In all the cell lines tested, W-7 inhibited EGF-stimulated Akt activation (Fig. 4, A and B, WB: P-Akt S-473). W-7 (30 μ M) also specifically inhibited insulin- or FBS-induced Akt activation in Myc83 cells (Fig. 4C, WB: P-Akt S-473). These observations suggest that calcium/calmodulin is a common regulator of Akt activation, irrespective of c-Myc expression status, species, tumorigenicity, and survival ligands in a variety of mammary epithelial cell models.

Calmodulin-mediated Cell Survival Does Not Depend on Calmodulin Kinase Kinase and Calmodulin Kinase II or III.—To determine whether calmodulin mediates Akt activation via calmodulin kinase(s), we tested specific inhibitors of calmodulin kinases. Serum-starved Myc83 cells were incubated with 100 ng/ml STO-609 for 6 h, 10 μ M KN-62 for 2 h, or 10 μ M Rottlerin for 2 h, followed by stimulation with 10 nM EGF for 3 min. STO-609 is a potent inhibitor of calmodulin kinase kinase (61, 62), which is an upstream activator of calmodulin kinase I and calmodulin kinase IV (6, 63). KN-62 (inactive analogue KN-92) inhibits calmodulin kinase II (64, 65), and Rottlerin (66) is an inhibitor of calmodulin kinase III (also known as EF-2 kinase). None of these compounds inhibited EGF-induced activation of Akt in Myc83 cells (Fig. 5, A–C, WB: P-Akt S-473),

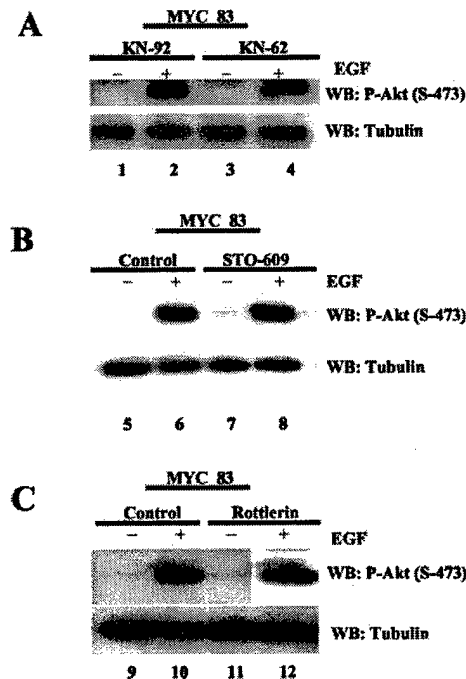


FIG. 5. Calmodulin kinases do not transduce EGF signal(s) to Akt. Myc83 cells were serum-starved overnight and incubated with 10 μ M KN-62 (lanes 3 and 4) or inactive analogue KN-92 (lanes 1 and 2) for 2 h (A), 100 ng/ml STO-609 (lanes 7 and 8) or vehicle (lanes 5 and 6) for 6 h (B), and 10 μ M Rottlerin (lanes 11 and 12) or vehicle (lanes 9 and 10) for 2 h (C). Cells were stimulated with 10 nM EGF for 3 min, and lysates were probed for Akt activity (WB: P-Akt S-473) as described before. Each of the blots was reprobed with anti-tubulin antibody (WB: Tubulin).

Myc83 (data not shown), and Comma D cells (data not shown). Although calmodulin kinase III acts downstream of Akt kinase, and PDK-1-null cells have no calmodulin kinase III activity (67), we used Rottlerin to rule out the possibility of any feedback activation of Akt by calmodulin kinase III. These data suggest that neither calmodulin kinase kinase, calmodulin kinase II, nor calmodulin kinase III transduces EGF-induced, EGFR-originated, and calmodulin-mediated signals to Akt.

Calmodulin Antagonist Does Not Inhibit EGF-induced PI-3 Kinase Activation.—Our laboratory previously demonstrated that EGF-dependent survival signaling in Myc83 cells is PI-3 kinase-Akt dependent, because preincubation of cells with PI-3 kinase inhibitor LY294002, inhibited Akt activation, leading to apoptosis (33). Overexpression of constitutively active myr-Akt protected Myc83 cells from LY294002-induced apoptosis (33). Joyal *et al.* (28) have demonstrated that calmodulin binds to the p85 α subunit of PI-3 kinase in a calcium-dependent manner. However, this interaction did not produce any phosphatidylinositol 3,4,5-triphosphate and thus cannot recruit Pleckstrin homology domain-containing proteins, such as PDK1 or Akt, to the plasma membrane (10, 68). A calcium/calmodulin-mediated mechanism of PI-3 kinase (hVPS34) activation was reported recently that describes the mechanism that *Mycobacterium tuberculosis* employs to block phagosome maturation and to evade bactericidal agents (29). To investigate whether calcium/calmodulin-mediated Akt activation is linked to classic EGF-induced PI-3 kinase-Akt activation, mediated by phosphotyrosine-p85 subunits, we employed the specific EGFR tyrosine kinase inhibitor AG1478 (69), the calmodulin antagonist W-7, and the phospholipase C- γ inhibitor U-73122 in Myc83 cells. We then examined whether any of these pharmacological inhibitors affected the ability of p85 α regulatory subunit to be co-immunoprecipitated with an anti-phosphotyrosine antibody.

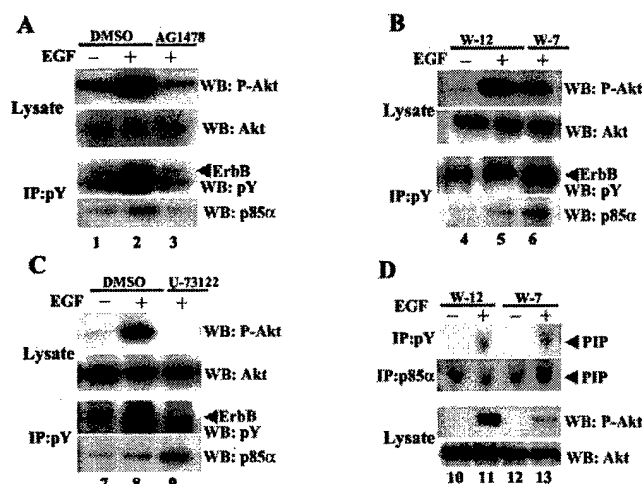


FIG. 6. Calmodulin antagonist W-7 has no effect on EGF-induced phosphotyrosine-associated PI-3 kinase activities. A, EGFR kinase inhibitor AG1478 inhibits both EGF-induced Akt activation and association of p85 α with phosphotyrosine. Serum-starved Myc83 cells were pre-incubated with either Me₂SO (DMSO) or 1 μ M of the specific EGFR tyrosine kinase inhibitor AG1478 for 30 min and then stimulated by EGF (lanes 2 and 3), as described previously. Lysates were immunoprecipitated by anti-pY antibody (clone 4G10), and immunoprecipitates were immunoblotted by anti-pY antibodies (clone 4G10) (WB: pY) and p85 α (WB: p85 α). An arrow on the pY immunoblot showed the position of ErbB. B and C, the calmodulin antagonist W-7 and the PLC- γ inhibitor U-73122 inhibit EGF-induced Akt activation but not the association of p85 α with phosphotyrosine. Myc83 cells were serum-starved and pre-incubated with 30 μ M W-12 or W-7 (lanes 4–6 B) or 10 μ M U-73122 (lanes 7–9 C) for 30 min and stimulated by EGF. Lysates were immunoprecipitated by anti-pY antibodies as described in A. Immunoprecipitates were immunoblotted for pY and p85 α (WB: pY, WB: p85 α , B and C). Representative lysates from each experiment were probed for the status of Akt activation and Akt (WB: P-Akt, WB: Akt, B and C) D, the calmodulin antagonist W-7 does not inhibit EGF-induced phosphotyrosine- and p85 α -associated PI-3 kinase activities *in vitro*. Immunoprecipitations were performed on Myc83 lysates treated with either W-12 (lanes 10 and 11) or W-7 (lanes 12 and 13) and stimulated with (lanes 11 and 13) or without EGF (lanes 10 and 12). *In vitro* PI-3 kinase assays were performed on immunoprecipitates, as described under "Experimental Procedures." Corresponding lysates were probed for their Akt activation status (WB: P-Akt).

Serum-starved Myc83 cells were treated with 1 μ M AG1478, 1 μ M U-73122, or 30 μ M W-7 for 30 min and then stimulated by EGF. Phosphotyrosine-containing proteins from the lysates were captured with an anti-phosphotyrosine antibody, followed by immunoblotting with anti-p85 α pAb. As shown in Fig. 6A, p85 α was co-immunoprecipitated, in an EGF-dependent manner, by anti-phosphotyrosine antibody, but not when cells were pre-incubated with AG1478 (WB: p85 α). Corresponding lysates from this experiment revealed that EGF-dependent Akt activation was strongly inhibited by AG1478 (Fig. 6A, WB: P-Akt). Myc83 cells express EGFR and ErbB2 (henceforth called ErbB) and do not express significant ErbB3 and ErbB4 as determined by Western blotting and cellular signaling (data not shown). Reprobing of the p-Tyr immunoprecipitates revealed that AG1478 significantly inhibited EGF-induced ErbB tyrosine phosphorylation (Fig. 6A, WB: pY). This demonstrates that classic phosphotyrosine-mediated, p85-dependent PI-3 kinase activation is operating in Myc83 cells. Because EGF-induced and calcium/calmodulin-dependent Akt activation require ErbB phosphotyrosine (such as EGFR Tyr-1173) to activate PLC- γ (required for calcium release), it was not possible to separate calcium/calmodulin-dependent Akt activation from phosphotyrosine-dependent, PI-3 kinase-mediated Akt activation by AG1478 treatment. In contrast, the PLC- γ inhibitor

U-73122 and the calmodulin antagonist W-7 could not inhibit EGF-dependent pull down of p85 α with anti-phosphotyrosine antibody (Fig. 6B, IP: pY/WB: p85 α and Fig. 6C, IP: pY/WB: p85 α), although corresponding lysates revealed strong inhibition in EGF-dependent Akt activity by each of these inhibitors (Fig. 6, B and C, WB: P-Akt). In fact, EGF-dependent p85 α binding to phosphotyrosine was increased in cells treated with W-7 (Fig. 6B, IP: pY/WB: p85 α) and U-73122 (Fig. 6C, IP: pY/WB: p85 α).

Calmodulin is known to bind directly to the epidermal growth factor receptor (EGFR) (70, 71), and both calmodulin and calmodulin kinase II were reported to inhibit EGFR kinase activity (70, 72). The calmodulin-binding domain at the juxtamembrane region of EGFR has also been mapped recently (73, 74). In agreement with these observations, W-7 potentiated EGF-induced ErbB tyrosine phosphorylation, probably as a result of up-regulation of EGFR tyrosine kinase activity (Fig. 6B, IP: pY/WB: pY). Because EGF-dependent p85 α association with ErbB is affected by neither W-7 nor U-73122, one could conclude that calmodulin, although associated with EGFR, has no positive effect on association of p85 to ErbB. This indirectly confirms that plasma membrane targeting of functional PI-3 kinase, composed of p85-p110 heterodimer, is not affected by U-73122 or W-7.

To confirm whether calmodulin exerts any effect at the PI-3 kinase level, *in vitro* PI-3 kinase assays were conducted on both anti-p85 α and anti-phosphotyrosine immunoprecipitates. EGF-induced PI-3 kinase activities, associated with anti-p85 α regulatory subunit and anti-phosphotyrosine immunoprecipitates, could not be inhibited by the calmodulin antagonist W-7 (Fig. 6D). We immunoblotted anti-calmodulin immunoprecipitates and calmodulin-Sepharose precipitates by a panel of p85 and pan-p85 antibodies; in every case, no EGF or calcium-dependent association of p85 regulatory subunit with calmodulin was observed (data not shown). We also performed an *in vitro* PI-3 kinase assay on anti-calmodulin immunoprecipitates and calmodulin-Sepharose precipitates, in an EGF- and calcium-dependent manner, respectively, and could detect no PI-3 kinase activity in either case (data not shown). Taken together, these data confirm that calmodulin does not affect targeting of either functional PI-3 kinase or EGF-induced phosphotyrosine-associated PI-3 kinase activities in Myc83 cells.

Calmodulin Forms a Complex with Akt in an EGF-dependent Manner—To investigate whether calmodulin forms complexes with Akt in an EGF-dependent manner, calmodulin was immunoprecipitated from serum-starved and EGF-stimulated Myc83 cells, and immunoprecipitates were probed for bound Akt. As shown in Fig. 7, Akt was co-immunoprecipitated with calmodulin in EGF-stimulated Myc83 cells, and this binding was abolished by pretreatment with W-7 (WB: Akt). Lysates from the same experiment revealed a parallel inhibition of Akt kinase activities (Fig. 7, WB: P-Akt S-473, WB: P-Akt T-308). These data suggest that calmodulin forms an EGF-dependent complex by either direct or indirect binding with Akt.

Calmodulin Antagonist W-7 Induces Apoptosis in c-Myc-over-expressing but Not in Normal Mammary Epithelial Cells—To investigate whether calcium/calmodulin-mediated Akt activation contributes to mammary epithelial cell survival, Myc83, Myc α 75, and Comma D cells were subjected to calmodulin antagonism by W-7, and cellular apoptosis was studied by PARP cleavage and Hoechst staining. In Myc83 and Myc α 75 cells, W-7 treatment induced significant PARP cleavage after 16 h that could be rescued by *N*-benzyloxycarbonyl-VAD-fluoromethyl ketone, a broad spectrum caspase inhibitor. This result confirmed that an early apoptotic program was initiated in Myc83 and Myc α 75 cells in response to W-7 (Fig. 8A). These

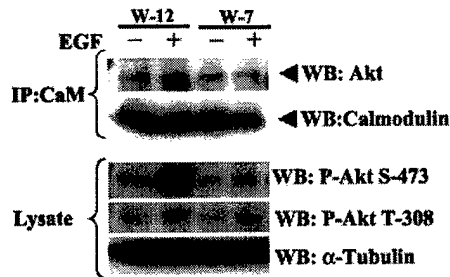
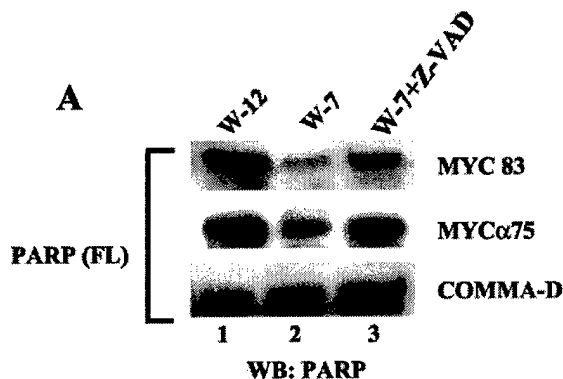


FIG. 7. Akt is co-immunoprecipitated with calmodulin in an EGF-dependent manner. EGF-induced co-immunoprecipitation of Akt with calmodulin. Serum-starved, semiconfluent Myc83 cells were pre-incubated with either 30 μ M W-12 (lanes 1 and 2) or W-7 (lanes 3 and 4) for 30 min and induced with or without 10 nM EGF for 3 min. Lysates were immunoprecipitated with an anti-calmodulin monoclonal antibody and then immunoprecipitates were immunoblotted for Akt (WB: Akt). The blot was reprobed with an anti-calmodulin pAb to demonstrate consistent immunoprecipitation (WB: Calmodulin). Corresponding lysates from this experiment were immunoblotted by anti-Phospho Akt (Ser-473) (WB: P-Akt S-473), anti-phospho Akt (Thr-308) (WB: P-Akt T-308), and by an anti-tubulin antibody (WB: α -Tubulin).



B

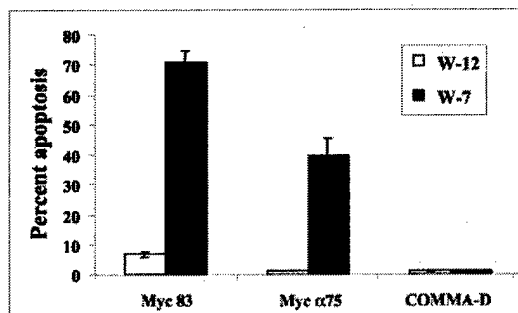


FIG. 8. Calmodulin antagonist induces apoptosis in Myc83, Myc α 75 but not in Comma D cells. A, semiconfluent growing cells were incubated with 30 μ M W-12 (lane 1) or W-7 (lane 2) or 30 μ M W-7, in the presence of 50 μ M *N*-benzyloxycarbonyl-VAD-fluoromethyl ketone (Z-VAD) (lane 3) for 16 h. Cells were trypsinized, lysed, and equal amount of total proteins were resolved in SDS-PAGE and immunoblotted by anti-PARP antibody (WB: PARP). B, semiconfluent growing cells were incubated with 30 μ M W-12 or W-7 for 36 h and all floating and adherent cells were stained with 10 μ g/ml of Hoechst 33258 dye (Sigma) for apoptotic analysis. For each replicate, at least 500 cells were counted and evaluated for the presence of condensed nuclei and overall apoptotic appearance. Each treatment was conducted in triplicate, and experiments were repeated three times. A representative experiment is presented.

carcinoma cells also showed a very high frequency of apoptosis, measured by Hoechst staining after 36 h of W-7 treatment (Fig. 8B). In contrast, Comma D cells did not undergo apoptosis by

W-7 in either of these assays (Fig. 8, A and B). Comma D cells previously showed a marked inhibition of EGF-induced Akt activation by W-7 (Fig. 3C). However, this compound had no effect on apoptosis. The simplest explanation for these opposite results is that additional event(s), independent of calmodulin-dependent Akt activation, are required to support survival mechanism of Comma D cells. A recent study demonstrated that immortalization of Comma D cells is independent of the EGF-PLC- γ kinase-Akt signaling cascade (75). Additional future experiments are required to determine the possible contribution of calmodulin to Comma D cell survival.

DISCUSSION

In this communication, we have presented evidence for the existence of a unique mechanism of EGF-induced and calcium/calmodulin-mediated survival in mouse mammary carcinoma cells. EGF-induced activation of Akt was shown to be a prime survival pathway of MMTV-c-Myc transgenic mammary tumor-derived epithelial cells (33). We have shown that EGF-induced and PLC- γ -mediated release of calcium from intracellular stores results in a calcium/calmodulin-dependent activation of Akt and survival of these cells. Calcium/calmodulin-regulated Akt activation in mammary epithelial cells is mediated neither by calmodulin kinases (6) nor directly by a PI-3 kinase-dependent mechanism(s), as described previously for neuronal cells (25). Calmodulin binds to Akt in an EGF-dependent manner, potentially targeting functional Akt to the plasma membrane for its subsequent activation by a PI-3 kinase-dependent mechanism. Calmodulin-mediated Akt activation, therefore, is indirectly linked to a phosphotyrosine-dependent, PI-3 kinase activation mechanism; perturbation of either mechanism by LY294002 (33) or calmodulin antagonist, W-7, induces apoptosis (in this study) in c-Myc-overexpressing mammary carcinoma cells. We also showed that calmodulin regulation of Akt kinase is common in a variety of mammary epithelial cells, irrespective of survival ligands (EGF, insulin, or FBS), c-Myc expression status, species (human or mouse), and tumorigenicity. Calmodulin antagonism specifically resulted in apoptosis of tumorigenic c-Myc-overexpressing mammary carcinoma cells but did not affect normal mammary gland-derived epithelial cells (i.e. Comma D), implying that calmodulin-mediated Akt activation is an integral part of the survival mechanism in certain tumorigenic cells.

Calmodulin is a universal calcium sensor and performs a myriad of biological functions including cell growth (76), cell cycle progression, proliferation (77, 78), trafficking (79), synaptic plasticity (80), and glucose transporter GLUT4 targeting to the plasma membrane (27, 81). Cellular incorporation of antisense calmodulin RNA and microinjection of calmodulin antibody leads to cell cycle arrest and inhibition of DNA synthesis (82). Recent publications have highlighted calmodulin's role in modulating cell survival, upstream of Akt kinase, both by PI-3 kinase-dependent or -independent mechanisms. In particular, calmodulin and calmodulin kinase kinase mediate membrane depolarization and, subsequently, cell survival in motor neurons and neuroblastoma cells by a PI-3 kinase-independent mechanism(s) (6, 83). Genetic studies also revealed that calcium/calmodulin, through calmodulin kinase, promotes *Saccharomyces cerevisiae* survival from pheromone-induced growth arrest (84). On the other hand, brain-derived neurotrophic factor- and neurotrophin-induced and calmodulin-mediated cell survival is considered to be mediated by PI-3 kinase-dependent Akt activation (25, 26). Although calmodulin was predicted to control generation of PI-3 kinase products in neuronal cells (25), the exact mechanism has not been addressed. Likewise, translocation of the GLUT4 glucose transporter to the plasma membrane in 3T3L1 adipocytes (27, 81) is regulated by

calcium (85), calmodulin, and Akt, although calmodulin's direct role could not be ascertained (27). In neuronal cells, a calmodulin antagonist inhibited Akt activation, and constitutively active Akt (gag-Akt) expressing neuronal cells escaped apoptosis induced by a calmodulin antagonist (25). In an analogous situation in 3T3-L1 adipocytes, a calmodulin antagonist inhibited insulin-induced Akt activation and GLUT4 translocation to the plasma membrane (27). An enhanced green fluorescent protein-Pleckstrin homology fusion protein also failed to translocate to the plasma membrane in the presence of a calmodulin antagonist. However, in both neuronal cells and 3T3-L1 cells, phosphotyrosine-associated *in vitro* PI-3 kinase was not inhibited by a calmodulin antagonist (25, 27). It was suggested that calcium/calmodulin is probably required for proper *in vivo* targeting of PI-3 kinase to its substrate and calmodulin antagonist inhibits this process. As a result, PI-3 kinase products, such as phosphatidyl inositol 3,4,5-triphosphates, are not produced, and Akt is not activated.

Our investigations are very similar to both of these observations, and we also observe EGF-induced Akt inactivation and apoptosis of Myc83 and Myc75 cells in the presence of W-7. Similar to neuronal and 3T3-L1 cells, phosphotyrosine- and p85 α -associated PI-3 kinase activities were not inhibited by a calmodulin antagonist *in vitro*. In contrast, in our experiments, calmodulin antagonism could not inhibit ligand-induced association between tyrosine phosphorylated ErbB and p85 α , implying that targeting of PI-3 kinase to the plasma membrane is not affected. Because membrane targeting of p85 (α/β) alone is not always sufficient for full PI-3 kinase/Akt activity (86), it is possible that the effect of calmodulin on Akt activation is at or distal to PI-3 kinase *in vivo*. Our *in vitro* lipid kinase data demonstrate that the calmodulin antagonist W-7 has no effect at the PI-3 kinase level. However, we observe a calmodulin-Akt association *in vitro* in an EGF-dependent manner that can be disrupted by W-7. This suggests that calmodulin probably performs a trafficking function for Akt by increasing Akt availability to PI-3 kinase products at the plasma membrane. Specific inhibition of either PI-3 kinase activation by LY294002 (33) or of Akt trafficking by a calmodulin antagonist (this study), thus inhibits Akt activation in these cells. A recent study demonstrated that glial cell line-derived neurotrophic factor-induced neuronal survival is mediated by calcium/calmodulin's association with PI-3 kinase, resulting in Akt activation (87). It was shown that calcium-dependent binding of calmodulin to the p85 regulatory subunit induces PI-3 kinase activation, resulting in Akt activation. Previous observations indicate that calmodulin-p85 interaction does not produce phosphatidyl inositol 3,4,5-triphosphate (28), a prime ligand for binding to Akt Pleckstrin homology domain for subsequent Akt activation. In view of this, it is not clear how calmodulin-p85 interaction and its associated PI-3 kinase activity resulted in Akt activation. Calmodulin is up-regulated in Myc83 cells (88), and constitutively activated Akt-expressing neuronal cells escape calmodulin antagonist-induced apoptosis (25). It is established that Akt needs to be plasma membrane-targeted for its activation. However, it is still not clear how Akt is transported to the plasma membrane from the cytoplasm. Because calmodulin forms a complex with Akt, it is more likely that calmodulin regulates Akt targeting and its consequent activation downstream of PI-3 kinase (Fig. 9).

EGF and related ligands, such as TGF α , β -cellulin, and amphiregulin, as well as the ErbB family receptors, have enormous influence on normal mammary development. In addition, dysregulation of either ligands or their receptors is frequently observed in breast cancer (89–92). Calmodulin is up-regulated in a variety of transgenic mouse mammary tumor models,

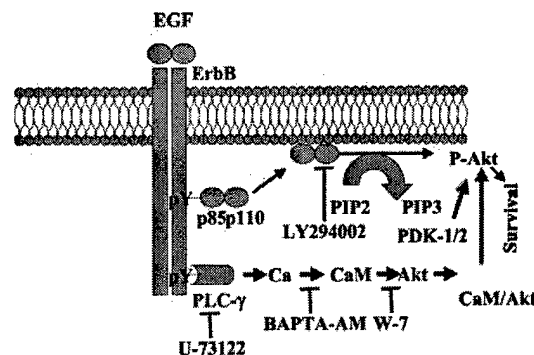


FIG. 9. A model of calcium/calmodulin-dependent Akt activation and survival in Myc83 cells. A probable model of calcium/calmodulin-induced Akt activation, and cell survival is presented. EGF-induced activation of ErbB tyrosine kinase produces phosphotyrosines, which can serve as binding ligands for Src homology 2-domain-containing proteins, such as the p85 regulatory subunit of PI-3 kinase and PLC- γ . The p85-p110 heterodimer is thus targeted to the plasma membrane, where PI-3 kinase is activated. Activated PLC- γ increases cytosolic calcium from intracellular stores. Calcium-bound activated calmodulin (CaM) associates with Akt and transports Akt to the plasma membrane, where Akt binds to PI-3 kinase products, such as phosphatidyl inositol-3,4,5-triphosphate (PIP3), and is subsequently activated by phosphorylation. Inhibition of PI-3 kinase (by LY294002), PLC- γ (by U-73122), chelation of intracellular calcium (by BAPTA-AM), and inactivation of calmodulin (by W-7) all result in Akt inhibition and apoptosis in Myc83 cells.

including MMTV-c-Myc (88). Our data demonstrate that calmodulin is a major contributory factor in Akt activation and cellular survival in c-Myc-overexpressing mouse mammary carcinoma cells. Furthermore, EGF-induced Akt activation was also strongly inhibited by W-7 in the human breast cancer cell line MCF-7 (data not shown), indicating that this mechanism is prevalent in human cancer cell lines that do not overexpress c-Myc. Previously, inhibition of calmodulin with W-7 and W-13 in the estrogen receptor-negative breast cancer cell line, MDA-MB-231, prevented colony formation in soft agar, suggesting that inhibition of calmodulin inhibits the transformation processes in certain human breast cancer cell lines independent of estrogen receptor status (93). Although we have shown that calmodulin forms a complex with Akt in an EGF-dependent manner, it is not known whether this interaction is direct or mediated by any auxiliary protein. The IQ motif is known to bind calmodulin in both a calcium-dependent and -independent manner (94). Based on hydropathy, hydrophobic residue, residue charge and mass, α -helical class, and position of particular residue, numerous calmodulin-binding proteins have been described previously (calcium.uhnres.utoronto.ca/ctdb/ctdb/home.html). In that context, Akt might potentially be a calmodulin-binding protein. In contrast, because EGF-induced Akt activation is also inhibited by staurosporine, it is probable that a staurosporine-sensitive, auxiliary protein kinase, other than PKC, mediates calmodulin's association to Akt. Our future investigations are directed toward investigating the interaction of Akt with calmodulin and associated survival signaling. These findings could have biologic relevance for defining the phenotype(s) of c-Myc-overexpressing breast cancer.

Acknowledgment—We thank Dr. Hallgeir Rui of LCCC for critical review of the manuscript.

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